Published in final edited form as:

Cell Immunol. 2011 ; 270(2): 95–102. doi:10.1016/j.cellimm.2011.04.011.

Host Derived Inflammatory Phospholipids Regulate *rahU* (PA0122) Gene, Protein, and Biofilm Formation in *Pseudomonas aeruginosa*

Jayasimha Rao^{*,‡}, Antonio DiGiandomenico[‡], Mykhaylo Artamonov[§], Norbert Leitinger[§], Ashok R. Amin^{*,†,‡}, and Joanna B. Goldberg[‡]

^{*}Research Department, Carilion Clinic; Roanoke, Virginia 24013

[†]Department of Biochemical Engineering, Virginia Tech; Blacksburg, Virginia 24061

[‡]Wake Forest University; Winston-Salem, NC 27109

[§]Cardiovascular Research Center, University of Virginia Health Sciences Center; Charlottesville, Virginia 22908

[‡]Department of Microbiology, University of Virginia Health Sciences Center; Charlottesville, Virginia 22908

Abstract

This study describes the role of “inflammatory” oxidative (Ox) phospholipids in regulation of *rahU* (PA0122) expression and biofilm formation in *Pseudomonas aeruginosa* (383) wild type (*rahU*⁺) and *rahU* mutant (*rahU*[−]). Functional analysis of RahU protein from *P. aeruginosa* in presence of Ox-phospholipids show: (a) LysoPC modulates RahU gene-protein expression in *rahU*⁺ cells; (b) *rahU* promoter activity is increased by lysoPC and inhibited by PAPC, Ox-PAPC and arachidonic acid; the latter inhibitory effect can be reversed by lysoPC, which was enzymatically derived from PAPC; (c) biofilm formation increased in *rahU*[−] cells as compared to *rahU*⁺; and (d) inhibition of *rahU* promoter activity by PAPC and AA (but not lysoPC) showed significantly augmented biofilm formation in *rahU*⁺ but not in *rahU*[−] cells. This study shows that host derived Ox-phospholipids affect *P. aeruginosa-rahU* gene and protein expression, which in turn modulates biofilm formation. The accompanying paper describes the role of RahU in eukaryotic-host cells.

Keywords

Pseudomonas; Inflammation; Phospholipids; Biofilm; Gene Expression

1. Introduction

Innate and adaptive immune systems and their inflammatory arm are primarily involved in combating infection [1;2]. Several cell types (e.g. leukocytes and lymphocytes) and soluble

© 2011 Elsevier Inc. All rights reserved.

[†]Corresponding author: Ashok R. Amin. PhD., Department of Biochemical Engineering, Virginia Tech; Blacksburg, Virginia 24061, Tel: 908 416 5739, Ashokmamin@yahoo.com.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

mediators (e.g. cytokines, chemokines and lipids) and other anti-inflammatory lipid mediators (such as lipoxins and others) [3–5] participate in this complex inflammatory process [6]. Eicosanoids play an integral role in both pro-inflammatory and inflammation resolution processes [7]. Phospholipids containing polyunsaturated fatty acids such as 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphotrylcholine (PAPC) are prone to oxidative modification (forming Ox-PAPC) [8]. Biological activity of Ox-PAPC is mediated by a variety of signal transduction mechanisms, which include elevated cAMP and, Ca^{++} levels, activation of MAPK, and small GTPase [9]. These phospholipids are also found to be accumulated in atherosclerotic vessels and influence LPS-induced cell injury [10]. Acute lung inflammation and injury caused by infection can lead to generation of lipid oxidation products such as lysoPC, PAPC and Ox-PAPC [11]. Ox-PAPC also stimulates angiogenesis via mechanisms involving IL-8 and COX-2 generated prostanoids. Ox-PAPC stimulates adhesion molecules in monocytes and endothelial cells [12]. Ox-PAPC harbors both pro-inflammatory and anti-inflammatory activity; however, the influence of these host-derived phospholipids on bacterial gene regulation remains largely unknown.

Pseudomonas aeruginosa is an opportunistic pathogen in humans [13–15]. It is versatile in its pathogenesis, as it can cause urinary, respiratory infections, and bacteremia particularly in individuals with compromised immune systems or in long term chemo treated cancer patients [16;17]. *P. aeruginosa* can exist in nonmucoid and/or mucoid forms in pathophysiological conditions such as cystic fibrosis (CF)-related lung infections. The phenotypically distinct mucoid mutants in chronically infected CF patients predominantly produce the exopolysaccharide alginate [18]. Several microbes have the ability to form a biofilm by aggregating and adhering to the surface of a structure (biotic or abiotic), along with secreted polymers [13]. *P. aeruginosa* displays similar properties and exhibits biofilm formation in pathophysiological conditions [19;20]. Chronic infection by *Pseudomonas* is normally associated with biofilm formation [21]. Bacterial biofilms are generally resistant to antibiotics and disinfectants, thus allowing microbes to survive in harsh conditions. Several gene products (including quorum sensing regulated genes of *Pseudomonas*) have been implicated in biofilm formation [22] and antibiotic resistance [23].

P. aeruginosa strains 383 (nonmucoid) and 2192 (mucoid) were isolated two days apart from a cystic fibrosis (CF) patient [24]. These two strains of *Pseudomonas* showed differentially expressed gene: PA0122 [25] coding for a hypothetical protein of unknown function. PA0122 mRNA levels showed higher expression in nonmucoid isolate (383) than mucoid (2192) [25]. This gene had homology to fungal and bacterial proteins belonging to the aerolysin family of proteins [26;27]. It showed 42% similarity to the fungal *Aspergillus* hemolysin protein (Asp-hemolysin) isolated from *Aspergillus fumigatus* [28]. Purified recombinant PA0122 protein from *E. coli* was shown to bind to Ox-LDL and lysoPC [25].

In the present paper, we investigate the putative role of Ox-phospholipids known to be involved in the host inflammatory process and their impact on PA0122 gene/protein expression and biofilm formation in *Pseudomonas*. We now refer to PA0122 gene as *rahU*, a Sanskrit word for “the seizer.” We show that *rahU* gene expression is also modulated by Ox-phospholipids and their products, and ultimately influences biofilm formation. These experiments further demonstrate the role of host Ox-phospholipids which are normally derived by host systems during microbial infections.

2. Materials and Methods

2.1. Reagents

Lysophosphatidylcholine; (LysoPC), 1-palmitoyl-2-arachidonoyl-sn-phosphatidylcholine; (PAPC) and oxidized- 1-palmitoyl-2-arachidonoyl-sn-phosphatidylcholine (Ox-PAPC) were

purchased from Avanti Polar Lipids (Alabaster, AL). PAPC was oxidized in air to obtain Ox-PAPC, whose formation was monitored by ESI-MS as described previously [29]. Phospholipase A₂ (PLA₂); arachidonic acid (AA); gentamicin (Gm); tetracycline (Tc); spectinomycin (Sp), and carbenicillin (Cb) were purchased from Sigma, (St. Louis, MO). 3,3',5,5'-tetramethylbenzidine; (TMB) in 0.01% H₂O₂ was obtained from Kirkegaard & Perry Laboratories, (Gaithersburg, MD) and 2-mm thick Kieselgel 60 plates were purchased from Merck (Whitehouse Station, NJ). Crystal violet (CV) solution (0.1%) was prepared from crystal violet powder (Thermo-Fisher, Pittsburgh, PA). All restriction enzymes and molecular biology reagents were procured from New England Biolabs, (Ipswich, MA). Tryptic soy agar plate; (TSA), blood agar plates (BA): 5% anticoagulated sheep's blood in TSA and Luria-Bertani medium (LB) were procured from Thermo-Fisher, Remel Products (Lenexa, KS) and, Bio-Rad (Hercules, CA) respectively.

2.2. Bacterial strain and growth conditions

Bacterial strains and plasmids used in this study are described in Supplemental Table 1. In this paper, *P. aeruginosa* nonmucoid strain 383, is designated as (*rahU*⁺). *P. aeruginosa* was cultured in LB medium at 37°C for 18 h, and typically inoculated for various experiments and adjusted to final absorbance of 0.01 (OD₆₀₀) in the fresh LB medium. *Escherichia coli* DH5α (Invitrogen, Carlsbad, CA) cells were used as recipients for plasmid constructs.

2.3. Construction of *rahU* mutant in *P. aeruginosa* strain 383

rahU gene was disrupted by inserting 1 kilo base (kb) gentamicin resistance (Gm^R) cassette in *P. aeruginosa* 383 as described [30]. This construct was utilized as an integration excision vector based on the allele replacement strategy that has been previously described [31;32]. Brief procedure and primers used in this study are described in the Supplemental Fig. 1. Insertion of the Gm^R cassette into *rahU* gene was confirmed by PCR and the resulting mutant strain was designated as *P. aeruginosa rahU*⁻ strain.

2.4. *rahU* promoter construction with *lacZ* reporter strain

A 410 base pair (bp) fragment, which consists of the *rahU* promoter and part of the open reading frame (orf) of *rahU* (PA0122) was constructed into mini-CTX-*lacZ* plasmids and integrated into the chromosome of *rahU*⁺ strain at *attB* site [33] in forward and reverse orientation respectively. The primers used in this study and brief procedure were described in the Supplemental Fig. 2A. The resultant Tc^S colonies were subcultured on TSA and Cb containing plates until pFLP2 was lost. The mini-CTX mediated integration at the *attB* site of *P. aeruginosa* chromosome and final constructs were denoted as *P. aeruginosa rahU*⁺p-*lacZ*-a, b, and c in the Supplemental Fig. 2B. These constructs were confirmed by PCR and sequencing, respectively.

2.5. Bioassay and normalization of *P. aeruginosa* for growth curve

All the *P. aeruginosa* strains (as mentioned in the Supplemental Table 1) were normalized at absorbance of 0.01 (OD₆₀₀) in fresh LB medium for 12 hours at shaking cultures at 37°C. Ox-phospholipid-treated and untreated cells were monitored for growth curve. Similarly, the growth curves of *rahU*⁺ and *rahU*⁻ were followed as mentioned above.

2.6. Western blotting analysis of *RahU*

Bacterial cells were collected at each time point for immunoblot analysis after normalizing them to OD₆₀₀ = 0.1, before subjecting them to equal volumes of Laemmli buffer for protein extraction. Equal volumes of each sample were loaded on 10% Tris-Tricine SDS-PAGE gels using the Criterion gel system (Bio-Rad). Transferred blots were probed with mouse polyclonal antibody dilution at 1:2000 used against r-RahU, followed with peroxidase

conjugated anti-mouse IgG (1:5000) to visualize the protein band using the ECL detection kit (GE Healthcare Biosciences, Pittsburgh, PA), as described previously [25].

2.7. Promoter analysis and β -galactosidase assay

β -galactosidase (β -gal) assay was performed by the method of Miller [34]. *P. aeruginosa* *rahU*⁺ promoter construct strains were grown in LB medium to an OD₆₀₀ = 0.02. Various modulators, including phospholipids, were added (at time 0) and the cells were collected at various subsequent time points, and normalized to an OD₆₀₀ = 0.1. Samples were assayed in triplicate. The β -gal activities were calculated by using formula: Miller units = $[\text{OD}_{420}/(t)(v) \text{ OD}_{600}] \times 1000$, where t is the time of reaction in minutes and v is the volume of the culture supernatant used in the assay (normalized at OD₆₀₀ = 0.1). All the experimental data were measured in Miller units and resulting values were subtracted from those obtained for controls *rahU*⁺-*lacZ*-b (reverse orientation) and *rahU*⁺-*lacZ*-c (empty plasmid), and expressed as a mean with Standard Deviation ($\pm$ SD) for each time point.

2.8. Phospholipase A₂ hydrolysis

Two mg of argon dried PAPC were re-suspended in 1 ml of phosphate buffered saline (PBS) at pH 7.0 containing 5 mM CaCl₂ with 5 units of PLA₂. The solution was mixed and incubated at 37°C for 45 min for hydrolysis. The hydrolyzed phospholipid was extracted with 1 ml of ethyl acetate containing 0.01% Butylated hydroxytoluene (BHT) after acidification with formic acid to pH 3.0 [35]. The resulting sample containing a mixture of lysoPC and AA was used in *rahU*⁺ promoter activity experiments. The control was set up with only AA with the *rahU*⁺ p-*lacZ*-b and c constructs.

2.9. Thin layer chromatography (TLC)

TLC was utilized for separation of mock PAPC from PLA₂-treated PAPC [36]. Briefly, PLA₂, treated and untreated samples were loaded onto preparative TLC plates (2-mm thick) using CHCl₃/MetOH/H₂O (10:5:1) as a solvent system. TLC separated lipids were viewed after treatment of the TLC plates with 10% copper sulfate in an 8.0% aqueous solution of orthophosphoric acid and subsequently heating them at 180°C as reported [37].

2.10. Electrospray ionization mass spectrometry (ESI-MS)

A Finnigan LSQ Classic mass analyzer (Thermo electron) was used for mass analysis and quantification of phospholipids. For flow-injection analysis (FIA) in the positive mode, phospholipids were dissolved in 100 μ L of MetOH/H₂O/ForAc (50:50:0.1). The mass to charge ratio was m/z 782 and 496 for PAPC and lysoPC, respectively [29].

2.11. Biofilm formation by *P. aeruginosa* of *rahU*⁺ wild type (WT) and *rahU*⁻ mutant

Biofilm formation was assayed using bacterial cells that were primed on TSA or BA plates for 18 h. Each independent experiment represented a single colony that was sub-cultured into LB medium and incubated at 37°C for 18 h. The overnight grown cultures were again sub-cultured in LB medium at a dilution of 1:100 (OD₆₀₀ of 0.01) and allowed to grow statically at 30°C for 24 h in glass tubes (Thermo-Fisher). The suspended cells were decanted from the glass tubes and the biofilm was stained with 0.1% crystal violet for 10 min. These tubes were excessively washed with distilled water, and allowed to dry at room temperature. The biofilm was solubilized with 33% glacial acetic acid in water and quantitated by measuring absorbance at OD₅₄₀ [38]. Statistical analysis was performed as described below.

2.12. Effect of different phospholipids on biofilm formation in *rahU*⁺ and *rahU*⁻ *P. aeruginosa* strains

The assay was performed in glass tubes, as described above in triplicates. Overnight cultures of *P. aeruginosa rahU*⁺ and *rahU*⁻ mutant cells were adjusted to 0.01 at OD₆₀₀ in fresh LB medium and allowed to grow to a cell density of 0.1 at OD₆₀₀, then treated with or without Ox-phospholipids; (1) without Ox-phospholipid; (2) with lysoPC (40 μM); (3) with PAPC (50 μg/ml); and (4) with Ox-PAPC (50 μg/ml) incubated statically at 30°C for 24 h. Biofilm was quantitated as described above.

2.13. Statistical Analysis

The statistical analysis was performed by using different approaches. *rahU*⁺ and *rahU*⁻ strains were compared using two-sided *t*-test for independent samples with pooled variance method for equal variances and Satterthwaite approximation for unequal variances. To ensure that the overall type I error was preserved as 0.05, the Bonferroni correction was also utilized. In addition, the inverse chi-square method of Fisher was also used to test for significance of combined results [39]. All three analyses showed statistically significant differences in biofilm formation between mutant versus wild type.

3. Results

In view of our previous observations [25] which established the physical binding of RahU to Ox-phospholipids, we investigated the physiological role of *rahU* gene and its protein in *P. aeruginosa* strain 383 (*rahU*⁺ and *rahU*⁻ disrupted mutant) in the presence of various Ox-phospholipids and their products. Aspects studied were: (a) regulation of *rahU* gene and protein expression and (b) biofilm formation.

3.1. Regulation of *RahU* protein expression in *P. aeruginosa* by lysoPC

Recent studies by our laboratory have shown the constitutive accumulation of RahU protein in the inner membrane of *Pseudomonas* and in spent medium [25]. RahU was constitutively produced and detected by Western blotting in the late log and early stationary phases of bacterial growth and r-RahU was found to bind to lysoPC (K_d : 10⁻⁶M) *in vitro* [25]. Although lysoPC had no effect on the growth of *P. aeruginosa* (Fig. 1), lysoPC increased RahU protein levels in cells at time periods between 5 to 10 h (Fig. 1). Thus, lysoPC induced a change in the kinetics of RahU accumulation (as shown by Western blots within the graph). This observation elicited an investigation on the regulation of *rahU* in *P. aeruginosa* in the presence of lysoPC and other phospholipids.

3.2. Effect of phospholipids on gene expression of *rahU* in *P. aeruginosa*

The *rahU* gene (PA0122) has the quorum sensing-*las* box in its promoter region [40]. To further investigate the regulation of *rahU*, we examined if inflammatory phospholipids influenced *rahU* gene expression in *P. aeruginosa*. A reporter construct harboring *rahU* gene segment (49 bases) with the *rahU* promoter, *las* box, and a β-gal reporter gene was constructed (Supplemental Fig. 2A and B). The basal β-gal activity confirmed that the *rahU*⁺-*lacZ*-b gene was constitutively expressed in a growth dependent manner (Fig. 2A). Transcriptional activation of the reporter construct was evident at 6 h of growth and peaked at about 10–11 h, consistent with the detection of the RahU protein at early and late stationary phases. Addition of 40 μM of lysoPC to the *rahU*⁺ cells showed a significant increase in *rahU* promoter activity. This observation was consistent with the early accumulation of RahU protein in lysoPC treated cells.

To further elucidate the role of lysoPC on *rahU* gene expression in *P. aeruginosa*, we examined the effect of other phospholipids which harbor lysoPC as a structural component.

1-palmitoyl-2-arachidonoyl-sn-phosphatidylcholine (PAPC) is a naturally occurring phospholipid containing (polyunsaturated) arachidonic acid and lysoPC [41]. Surprisingly, incubation of *P. aeruginosa* with PAPC and Ox-PAPC inhibited the *rahU*⁺-*lacZ*-b reporter activity when compared to its basal activity (Fig. 2B). Since lysoPC augmented the basal promoter activity in *P. aeruginosa*, reconstitution experiments were designed to identify a potential inhibitory motif in PAPC, lysoPC+AA. Fig. 2C depicts the inhibition of *rahU*⁺-*lacZ*-b by 50 µg/ml of free AA as compared to the promoters' basal activity. However, this inhibitory activity could be restored after 6 h of growth if the cells were concurrently supplemented with 50 µg/ml of lysoPC at 0 h. These results suggest that the inhibitory activity of PAPC on *rahU* gene expression may reside in AA. To further dissect the lipid structure-function relations in this signaling mechanism, we incubated PAPC with PLA₂ to release both AA and lysoPC and maintained these components as a mixture. Further, we also allowed PAPC to undergo "auto oxidation" for 12 h before it was tested in the promoter assay as "mock PAPC". Fig. 2D depicts TLC analysis of these products, whose identities were also confirmed by mass spectral analysis (data not shown). Fig. 2D also shows the accumulation of lysoPC in the "mock PAPC" solution (lane 1) and generation of AA and lysoPC (lane 2) on treatment of PAPC with PLA₂. Fig. 2E shows the basal *rahU*⁺-*lacZ*-b promoter activity in the presence of AA and PAPC, respectively. Addition of "mock PAPC" and/or PLA₂ treated PAPC significantly augmented the *rahU*⁺-*lacZ*-b activity at 5 h of *P. aeruginosa* growth.

3.3. Construction and characterization of *rahU*⁺ and *rahU*⁻ strains

We examined the role of the *RahU* gene and protein. The *rahU* gene was disrupted by the insertion of a gentamicin resistance cassette in *P. aeruginosa rahU*⁺ strain to generate a *RahU* defective mutant (*rahU*⁻). The lack of *rahU* mRNA and its protein product in *rahU*⁻ is shown in Supplemental Fig. 3AI to AIV and 3B. No difference in the growth pattern of *rahU*⁺ and *rahU*⁻ strains was observed when grown in LB media (Supplemental Fig. 3B).

3.4. Regulation of biofilm by *rahU*⁺ *rahU*⁻ cells

We examined biofilm formation in *rahU*⁺ and *rahU*⁻ cells. Fig. 3A depicts the quantification of biofilm formation in *rahU*⁺ and *rahU*⁻ cells that were primed on TSA (as seen in experiments 1–4) and on blood agar (as seen in experiments 5–7). Both the growth conditions showed similar levels of basal biofilm formation. However, the *rahU*⁻ mutant strain showed significant augmentation of biofilm formation as compared to *rahU*⁺ cells (Supplemental Fig. 3AIV and 3A). Furthermore, Fig. 3A showed that three different statistical analyses (2-sided *t*-tests, Satterthwaite approximation and inverse chi-square method of Fisher) revealed that *rahU*⁻ generated significantly higher levels of biofilm than *rahU*⁺.

3.5. Regulation of biofilm formation in *rahU*⁺ and *rahU*⁻ in by phospholipids

In view of the increased biofilm formation in *rahU*⁻ cells and the differential modulation of the *rahU* promoter activity by different phospholipids in *rahU*⁺ cells, we further examined the role of phospholipids on *rahU* mediated biofilm formation in these bacterial strains (Fig. 3B). It should be noted that the influence of gene expression by phospholipids was first evident between 6–12 h of growth as described in Fig. 2. We therefore examined the influence of phospholipids on biofilm accumulation at 12–16 h. LysoPC, which increased *RahU* protein accumulation (Fig. 1) and promoter activity in *P. aeruginosa* (Fig. 2A), had no significant effect on biofilm formation (at 12–16 h) in either *rahU*⁺ or *rahU*⁻ cells when compared to cells that were not exposed to LysoPC (Fig. 3B). Addition of PAPC or AA to *rahU*⁺ cultures, which decreased *rahU* gene expression (at 6–12 h), showed a significant (1 to 2 fold) increase in biofilm formation as compared to non-treated cells (Fig. 3B). No

significant effect on biofilm formation was observed when *rahU*⁻ cells were exposed to PAPC or AA (as compared to corresponding baseline levels).

4. Discussion

Previous studies have demonstrated a pivotal role of phospholipids in inflammation and innate immunity in eukaryotic systems [42]. Among these, specifically PAPC, lysoPC, AA and their products have been reported to modulate various cellular functions in eukaryotic systems, including modulation of adhesion molecules, receptor signaling, gene expression, and chemotaxis [12;43–45]. We have recently identified a protein, RahU, produced by *P. aeruginosa*, which binds to Ox-LDL and lysoPC [25]. Previous studies have shown that phosphatidyl inositol (PI) and phosphatidylserine (PS) bind to *P. aeruginosa*, possibly modulating bacterial adhesion and virulence factors [46]; and monopalmitoylphosphatidic acid inhibits expression of several secreted virulence factors, including alginate, pyoverdine, elastase and LasA from *P. aeruginosa* (PAO1) [47]. The present studies show a significant change in the early accumulation of RahU induced by lysoPC, which is likely due to modulation of gene expression and possibly protein turnover in *P. aeruginosa*. These observations are consistent with the ability of phospholipids to interact with bacterial cells as described above. Furthermore other host derived molecules such as IFN- γ have previously been reported to signal to *Pseudomonas* membrane protein [48]. Our results suggest that lysoPC, in its free form and/or as a (lysoPC+RahU) complex, modulates *rahU* gene expression, thus demonstrating the effect of host derived lysoPC on gene and protein expression in *P. aeruginosa* (*rahU*⁺ cells). This report establishes a role for inflammatory Ox-phospholipids in modulating *rahU* gene expression in *rahU*⁺ *P. aeruginosa* strain (Fig. 2). Although these experiments suggest that the level of *rahU* gene expression was dependent upon the structure of the phospholipids, the exact structural–functional relationship remains to be elucidated. It should be noted that the Ox-phospholipid modulator (e.g. lysoPC) of the *rahU* gene is also a ligand of the RahU protein [25], thus creating a potential feedback loop that might regulate *rahU* gene expression and other functions of the RahU protein. Earlier studies have shown that phospholipids may trigger secretion of flagellin in *Salmonella typhi* via cAMP [49]. Physiological characteristics of *rahU*⁺ and *rahU*⁻ cells were not markedly different in terms of metabolic requirements such as carbon utilization (data not shown). Besides protecting microorganisms from innate immune functions, increased biofilm formation increases the organisms susceptibility to mutagenesis, thus possibly affecting antibiotic sensitivity [50]. There were no significant differences in the antibiotic sensitivity of *rahU*⁺ and *rahU*⁻ cells (besides the mutant harboring the gentamicin resistance cassette). These experiments also suggest that the basic characteristics of *rahU*⁻, including growth kinetics, remained unaltered. *P. aeruginosa* biofilm formation is known to be associated with the chronic phase of infection, which is typically seen in cystic fibrosis patients, ventilated patients, urinary catheter infected patients, and burn patients [51;52]. Biofilm formation has been reported to be coordinately regulated by various quorum-sensing genes in microorganisms [53]. *rahU* has been reported to be a quorum sensing, regulated gene which harbors a quorum sensing motif *las* in its promoter [40]. These observations support a potential role of *rahU* in biofilm formation.

The innate immune system depends on phagocytes, including neutrophils and macrophages, for engulfing and killing microorganisms. This is particularly true in the case of *P. aeruginosa* since neutropenic mice are highly susceptible to infection when compared to their wild type counterparts [54]. This defensive mechanism is very effective against individual planktonic organisms as compared to biofilms. In addition, the presence of exopolysaccharides on the surface of *P. aeruginosa* is also known to prevent complement deposition on the bacterial surface, thus effectively reducing the ability of neutrophils and macrophages to promote bacterial killing [55]. Other mechanisms may include the decreased

oxygen-dependent (nitric oxide and superoxide) and oxygen-independent host-neutrophil responses (lysozyme, lactoferrin) to *P. aeruginosa* biofilms (<http://www.microbemagazine.org/index.php/02-2009-home/58-bacterial-biofilms-resist-key-host-defenses>). Therefore, it seems plausible to suggest that induction of mucoidy in *P. aeruginosa* by host derived phospholipids is an adaptation by the organism to thwart the intense inflammatory environment that ensues in an infected lung. Bacterial biofilm may act as fuel during chronic inflammatory state via innate and acquired immune responses. Phospholipids containing components such as pulmonary surfactants formed by lung cells modulate the function of immune cells in the lungs and participate in the first line of host-defense reactions [56]. Pulmonary surfactants may foster persistent lung infections by biofilm producing *Pseudomonas* in cystic fibrosis patients.

These investigations suggested that the *rahU* gene and/or protein are prerequisite(s) for modulation of biofilm formation in *P. aeruginosa* by inflammatory Ox-phospholipids. Our results assign an additional function to PAPC and AA in host-bacterial interaction. It is tempting to speculate that these molecules (RahU and or phospholipids) might be utilized as sensors of inflammation and/or innate immunity by the bacteria via extracellular ligands/receptors (RahU) to modify biofilm formation and host-bacterial interactions. These studies also highlight the possible feedback regulation of biofilm formation, based on the presence of RahU in the local environment, since this protein functions as a high-affinity ligand for Ox-LDL (10^{-9} M) and lysoPC (10^{-6} M), respectively [25]. These studies show the emergence of two types of behavior: (a) RahU intercellular signaling and (b) the formation of surface-associated communities: biofilms. Host-derived factors such as lactoferrin, Tamm-Horfall glycoprotein (from urine), and IFN- γ have been reported to influence biofilm formation and virulence in *Pseudomonas* [57].

In summary, the present study proffers the following hypothesis: *rahU* not only regulates *P. aeruginosa* phenotype (with respect to biofilm formation), but also adapts and modulates cellular functions based on the Ox-phospholipids known to be derived from host cells (Fig. 4). This potential “inflammation sensing” apparatus may facilitate the opportunistic behavior of *Pseudomonas* in host-bacterial interactions. It is well known that *Pseudomonas*, which co-exists with the host, continually senses the environment and adapts itself to various phenotypic forms and may augment its pathogenesis as evident in cystic fibrosis, AIDS and burn patients, and in conditions when the immune system is compromised.

In conclusion, these studies for the first time demonstrate a pathogen derived protein that assists in “seizing” opportunities at the molecular level to perform various pleiotropic functions consistent with *Pseudomonas* characteristics. The accompanying paper describes the expanding functions of RahU in host cells: macrophages and monocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Bob Herbertson for some of the data and statistical analysis in this paper. We thank Dale Grgrurich for his assistance with metabolic analysis and antibiotic susceptibility assay in the Carilion Clinic Laboratory with various strains of *P. aeruginosa*. We would like to thank Marjorie Gowdy and Dr. Smita Palejwala for their helpful comments and editing on this manuscript. This study was supported by a grant from the University of Virginia Funding for Excellence in Science and Technology (FEST) Program to J. B. G., the National Institutes of Health (NIH) (R21- AI53842) to J. B. G., NIH grant R01 HL084422 for N. L., and RAP3 support from Carilion Clinic to R.J and A.R.A.

ABBREVIATIONS

<i>rahU</i>⁺	Wild type <i>P. aeruginosa</i> strain 383, expressing PA0122 protein
<i>rahU</i>⁻	Mutant <i>P. aeruginosa</i> strain 383, lacking expression of PA0122 protein
LysoPC	lysophosphatidylcholine; 1-hexanoyl-2-hydroxy-sn-glycero- 3-phosphocholine
PAPC	1-palmitoyl-2-arachidonoyl-sn-phosphatidylcholine
Ox-PAPC	oxidized-1-palmitoyl-2-arachidonoyl-sn-phosphatidylcholine
PLA₂	phospholipase A ₂
AA	arachidonic acid
Gm	gentamicin
Tc	tetracycline
Sp	spectinomycin
Cb	Carbenicillin
TMB	3,3',5,5'-tetramethylbenzidine
CV	crystal violet
TSA	tryptic soya agar plate
BA	blood agar plates
LB	Luria–Bertani medium
RahU	a Sanskrit word for “the seizer”
Asp-hemolysin	<i>Aspergillus</i> -hemolysinprotein
BHT	Butylated hydroxytoluene

References

1. Kumagai Y. Pathogen recognition by innate receptors. *Infect Chemother.* 2008; 14:86–92.
2. Ishii KJ, Koyama S, Nakagawa A, Coban C, Akira S. Host innate immune receptors and beyond: making sense of microbial infections. *Cell Host Microbe.* 2008; 12:352–363. [PubMed: 18541212]
3. Kobayashi SD, Voyich JM, Burlak C, DeLeo FR. Neutrophils in the innate immune response. *Arch Immunol Ther Exp.* 2005; 53:505–517.
4. Machen TE. Innate immune response in CF airway epithelia: hyperinflammatory? *Am J Physiol Cell Physiol.* 2006; 291:218–230.
5. Netea MG, Kullberg BJ, Van der Meer JW. Proinflammatory cytokines in the treatment of bacterial and fungal infections. *BioDrugs.* 2004; 18:9–22. [PubMed: 14733604]
6. Lawrence T, Willoughby DA, Gilroy DW. Anti-inflammatory lipid mediators and insights into the resolution of inflammation. *Nat Rev Immunol.* 2002; 2:787–795. [PubMed: 12360216]
7. Serhan CN, Yacoubian S, Yang R. Anti-inflammatory and proresolving lipid mediators. *Annu Rev Pathol.* 2008; 3:279–312. [PubMed: 18233953]
8. Leitinger N. Oxidized phospholipids as triggers of inflammation in atherosclerosis. *Mol Nutr Food Res.* 2005; 49:1063–71. [PubMed: 16270279]
9. Cole G, Subbanagounder AL, Mukhopadhyay S, Berliner JA, Vora DK. Oxidized phospholipid-induced endothelial cell/monocyte interaction is mediated by a cAMP-dependent R-Ras/PI3-kinase pathway. *Arterioscler, Thromb Vasc Biol.* 2003; 1:1384–1390. [PubMed: 12805072]

10. Bochkov VN, Kadl A, Huber J, Gruber F, Binder BR, Leitinger N. Protective role of phospholipid oxidation products in endotoxin-induced tissue damage. *Nature*. 2002; 419:77–81. [PubMed: 12214235]
11. Nonas S, Miller I, Kawkitinarong K, Chatchavalvanich S, Gorshkova I, Bochkov VN, Leitinger N, Natarajan V, Garcia JGN, Birukov KG. Oxidized Phospholipids Reduce Vascular Leak and Inflammation in Rat Model of Acute Lung Injury. *Am J Respir Crit Care Med*. 2006;200511-1737OC.
12. Birukov KG, Leitinger N, Bochkov VN, Garcia JG. Signal transduction pathways activated in human pulmonary endothelial cells by OxPAPC, a bioactive component of oxidized lipoproteins. *Microvasc Res*. 2004; 67(1):18–28. [PubMed: 14709399]
13. Hall-Stoodley L, Stoodley P. Biofilm formation and dispersal and the transmission of human pathogens. *Trends Microbiol*. 2005; 13(1):7–10. [PubMed: 15639625]
14. Son MS, Matthews WJ Jr, Kang Y, Nguyen DT, Hoang TT. In Vivo Evidence of *Pseudomonas aeruginosa* Nutrient Acquisition and Pathogenesis in the Lungs of Cystic Fibrosis Patients. *Infect Immun*. 2007; 75:5313–5324. [PubMed: 17724070]
15. Miller RM, Tomaras AP, Barker AP, Voelker DR, Chan ED, Vasil AI, Vasil ML. *Pseudomonas aeruginosa* Twitching Motility-Mediated Chemotaxis towards Phospholipids and Fatty Acids: Specificity and Metabolic Requirements. *J Bacteriol*. 2008; 190:4038–4049. [PubMed: 18390654]
16. Sadikot RT, Blackwell TS, Christman JW, Prince AS. Pathogen-host interactions in *Pseudomonas aeruginosa* pneumonia. *Am J Respir Crit Care Med*. 2005; 171:1209–1223. [PubMed: 15695491]
17. Davies JC. *Pseudomonas aeruginosa* in cystic fibrosis: pathogenesis and persistence. *Paediatr Respir Rev*. 2002; 3:128–134. [PubMed: 12297059]
18. Parsek MR, Matthew R, Singh PK. Bacterial biofilms: an emerging link to disease pathogenesis. *Annu Rev Microbiol*. 2003; 57(677):701.
19. Kirov SM, Webb JS, O'May CY, Reid DW, Woo JKK, Rice SA, Kjelleberg S. Biofilm differentiation and dispersal in mucoid *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. *Microbiology*. 2007; 153:3264–3274. [PubMed: 17906126]
20. Mikkelsen H, Duck Z, Lilley KS, Welch M. Interrelationships between Colonies, Biofilms, and Planktonic Cells of *Pseudomonas aeruginosa*. *J Bacteriol*. 2007; 189:2411–2416. [PubMed: 17220232]
21. Wagner VE, Iglewski BH. *P. aeruginosa* Biofilms in CF Infection. *Clin Rev Allergy Immunol*. 2008; 35(3):124–134. [PubMed: 18509765]
22. Landry RM, An D, Hupp JT, Singh PK, Parsek MR. Mucin-*Pseudomonas aeruginosa* interactions promote biofilm formation and antibiotic resistance. *Mol Microbiol*. 2006; 59(1):142–151. [PubMed: 16359324]
23. Hentzer M, Teitzel GM, Balzer GJ, Heydorn A, Molin S, Givskov M, Parsek MR. Alginate overproduction affects *Pseudomonas aeruginosa* biofilm structure and function. *J Bacteriol*. 2001; 183(5395):5401.
24. Hanna SL, Sherman NE, Kinter MT, Goldberg JB. Comparison of proteins expressed by *Pseudomonas aeruginosa* strains representing initial and chronic isolates from a cystic fibrosis patient: an analysis by 2-D gel electrophoresis and capillary column liquid chromatography-tandem mass spectrometry. *Microbiology*. 2000; 146:2495–2508. [PubMed: 11021925]
25. Rao J, DiGiandomenico A, Unger J, Bao Y, Polanowska-Grabowska RK, Goldberg JB. A novel oxidized low-density lipoprotein-binding protein from *Pseudomonas aeruginosa*. *Microbiology*. 2008; 154:654–665. [PubMed: 18227268]
26. Kumagai T, Tsutsumi H, Ogawa N, Naito S, Ebina K, Yokota K, Nagata K. Oxidized low-density lipoprotein-binding specificity of the Asp-hemolysin-related synthetic peptides from *Aspergillus fumigatus*. *Biol Pharm Bull*. 2006; 29(11):2181–2186. [PubMed: 17077511]
27. Kudo Y, Ootani T, Kumagai T, Fukuchi Y, Ebin K, Yokota K. A novel oxidized low-density lipoprotein-binding protein, Asp-hemolysin, recognizes lysophosphatidylcholine. *Biol Pharm Bull*. 2002; 25(6):787–90. [PubMed: 12081148]
28. Marchler-Bauer A, Bryant SH. CD-Search: protein domain annotations on the fly. *Nucleic Acids Res*. 2004; 32(Web Server issue):W327–31. [PubMed: 15215404]

29. Watson AD, Leitinger N, Navab M, Faull KF, Horkko S, Witztum JL, Palinski W, Schwenke D, Salomon RG, Sha W, Subbanagounder G, Fogelman AM, Berliner JA. Structural Identification by Mass Spectrometry of Oxidized Phospholipids in Minimally Oxidized Low Density Lipoprotein That Induce Monocyte/Endothelial Interactions and Evidence for Their Presence in Vivo. *J Biol Chem.* 1997; 272:13597–13607. [PubMed: 9153208]
30. Schweizer HP. Allelic exchange in *Pseudomonas aeruginosa* using novel ColE1-type vectors and a family of cassettes containing a portable oriT and the counter-selectable *Bacillus subtilis* sacB marker. *Mol Microbiol.* 1992; 6(9):1195–204. [PubMed: 1588818]
31. Schweizer HP, Hoang TT. An improved system for gene replacement and xylE fusion analysis in *Pseudomonas aeruginosa*. *Gene.* 1995; 158:15–22. [PubMed: 7789804]
32. Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene.* 1998; 212:77–86. [PubMed: 9661666]
33. Becher A, Schweizer HP. Integration-proficient *Pseudomonas aeruginosa* vectors for isolation of single-copy chromosomal *lacZ* and *lux* gene fusions. *Biotechniques.* 2000; 29:948–50. 952. [PubMed: 11084852]
34. Miller, JH. *Experiments in Molecular Genetics.* Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY: 1972.
35. Watson AD, Subbanagounder G, Welsbie DS, Faull KF, Navab M, Jung ME, Fogelman AM, Berliner JA. Structural Identification of a Novel Pro-inflammatory Epoxyisoprostane Phospholipid in Mildly Oxidized Low Density Lipoprotein. *J Biol Chem.* 1999; 274:24787–24798. [PubMed: 10455151]
36. Bochkov VN, Mechtcheriakova D, Lucerna M, Huber J, Malli R, Graier WF, Hofer E, Binder BR, Leitinger N. Oxidized phospholipids stimulate tissue factor expression in human endothelial cells via activation of ERK/EGR-1 and Ca(++)/NFAT. *Blood.* 2002; 99:199–206. [PubMed: 11756172]
37. Gruber F, Oskolkova O, Leitner A, Mildner M, Mlitz V, Lengauer B, Kadl A, Mrass P, Kronke G, Binder BR, Bochkov VN, Leitinger N, Tschachler E. Photooxidation Generates Biologically Active Phospholipids That Induce Heme Oxygenase-1 in Skin Cells. *J Biol Chem.* 2007; 282:16934–16941. [PubMed: 17449870]
38. O'Toole G, Kaplan HB, Kolter R. Biofilm formation as microbial development. *Annu Rev Microbiol.* 2000; 54:49–79. [PubMed: 11018124]
39. Fisher, RA. *Statistical Methods for Research Workers.* Oliver and Boyd; London: 1932.
40. Wagner VE, Bushnell D, Passador L, Brooks AI, Iglewski BH. Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. *J Bacteriol.* 2003; 185(7):2080–95. [PubMed: 12644477]
41. Leitinger N, Tyner TR, Oslund L, Rizza C, Subbanagounder G, Lee H, Shih PT, Mackman N, Tigyi G, Territo MC, Berliner JA, Vora DK. Structurally similar oxidized phospholipids differentially regulate endothelial binding of monocytes and neutrophils. *P Natl Acad Sci USA.* 1999; 96(21):12010–12015.
42. Leitinger N. Oxidized phospholipids as modulators of inflammation in atherosclerosis. *Curr Opin Lipidol.* 2003; 14:421–430. [PubMed: 14501580]
43. Han KH, Hong KH, Ko J, Rhee KS, Hong MK, Kim JJ, Kim YH, Park SJ. Lysophosphatidylcholine up-regulates CXCR4 chemokine receptor expression in human CD4 T cells. *J Leukoc Biol.* 2004; 76:195–202. [PubMed: 15178707]
44. Sakai M, Miyazaki A, Hakamata H, Sato Y, Matsumura T, Kobori S, Shichiri M, Horiuchi S. Lysophosphatidylcholine Potentiates the Mitogenic Activity of Modified LDL for Human Monocyte Derived Macrophages. *Arterioscler Thromb Vasc Biol.* 1996; 16:600–605. [PubMed: 8624783]
45. Kreuzer J, Denger S, Jahn L, Bader J, Ritter K, von Hodenberg E, Kubler W. LDL Stimulates Chemotaxis of Human Monocytes Through a Cyclooxygenase-Dependent Pathway. *Arterioscler Thromb Vasc Biol.* 1996; 16:1481–1487. [PubMed: 8977452]

46. Panjwani N, Zhao Z, Raizman MB, Jungalwala F. Pathogenesis of corneal infection: binding of *Pseudomonas aeruginosa* to specific phospholipids. *Infect Immun*. 1996; 64:1819–1825. [PubMed: 8613396]
47. Laux DC, Corson JM, Givskov M, Hentzer M, Moller A, Wosencroft KA, Olson JC, Krogfelt KA, Goldberg JB, Cohen PS. Lysophosphatidic acid inhibition of the accumulation of *Pseudomonas aeruginosa* PAO1 alginate, pyoverdine, elastase and LasA. *Microbiology*. 2002; 148:1709–1723. [PubMed: 12055291]
48. Wu L, Estrada O, Zaborina O, Bains M, Shen L, Kohler JE, Patel N, Musch MW, Chang EB, Fu YX, Jacobs MA, Nishimura MI, Hancock REW, Turner JR, Alverdy JC. Recognition of Host Immune Activation by *Pseudomonas aeruginosa*. *Science*. 2005; 309:774–777. [PubMed: 16051797]
49. Subramanian N, Qadri A. Lysophospholipid sensing triggers secretion of flagellin from pathogenic salmonella. *Nat Immunol*. 2006; 7:583–589. [PubMed: 16648855]
50. Livermore DM, Struelens M, Amorim J, Baquero F, Bille J, Canton R, Henning S, Gattermann S, Marchese A, Mittermayer H, Nonhoff C, Oakton KJ, Praplan F, Ramos H, Schito GC, Van Eldere J, Verhaegen J, Verhoef J, Visser MR. Multicentre evaluation of the VITEK 2 Advanced Expert System for interpretive reading of antimicrobial resistance tests. *J Antimicrob Chemother*. 2002; 49:289–300. [PubMed: 11815570]
51. Al Aloul M, Crawley J, Winstanley C, Hart CA, Ledson MJ, Walshaw MJ. Increased morbidity associated with chronic infection by an epidemic *Pseudomonas aeruginosa* strain in CF patients. *Thorax*. 2004; 59:334–336. [PubMed: 15047956]
52. Bodey GP, Bolivar R, Fainstein V, Jadeja L. Infections caused by *Pseudomonas aeruginosa*. *Rev Infect Dis*. 1983; 5(2):279–313. [PubMed: 6405475]
53. Hentzer M, Eberl L, Nielsen J, Givskov M. Quorum sensing: a novel target for the treatment of biofilm infections. *Biodrugs*. 2003; 17(4):241–50. [PubMed: 12899641]
54. Koh AY, Priebe GP, Ray C, Van Rooijen N, Pier GB. Inescapable Need for Neutrophils as Mediators of Cellular Innate Immunity to Acute *Pseudomonas aeruginosa*. *Pneumonia Infect Immun*. 2009; 77:5300–5310.
55. Pier GB, Coleman F, Grout M, Franklin M, Ohman DE. Role of alginate O acetylation in resistance of mucoid *Pseudomonas aeruginosa* to opsonic phagocytosis. *Infect Immun*. 2001; 69(3):1895–901. [PubMed: 11179370]
56. van Golde LM. Potential role of surfactant proteins A and D in innate lung defense against pathogens. *Biol Neonate*. 1995; 67(Suppl 1):2–17. [PubMed: 7647156]
57. Wu L, Estrada O, Zaborina O, Bains M, Shen L, Kohler JE, Patel N, Musch MW, Chang EB, Fu YX, Jacobs MA, Nishimura MI, Hancock REW, Turner JR, Alverdy JC. Recognition of Host Immune Activation by *Pseudomonas aeruginosa*. *Science*. 2005; 309:774–777. [PubMed: 16051797]
58. Hoang TT, Kutchma AJ, Becher A, Schweizer HP. Integration-proficient plasmids for *Pseudomonas aeruginosa*: site-specific integration and use for engineering of reporter and expression strains. *Plasmid*. 2000; 43(1):59–72. [PubMed: 10610820]

Research Highlights

- LysoPC modulates Pseudomonas protein- RahU
- Oxidized phospholipids regulate microbial gene expression
- Pseudomonas protein: RahU regulates biofilm formation

Figure 1.

LysoPC, which augments *RahU* production in *P. aeruginosa* *rahU*⁺ strain, was cultured in LB medium with (▲—▲) or without (□—□) 40 μ M lysoPC as described in Materials and Methods. Total protein lysates from *rahU*⁺ *P. aeruginosa* were prepared at different growth time points for immunoblot analysis. All individual bacterial samples were normalized to OD₆₀₀ = 0.1 prior to protein preparation, and equal volumes (representing ~ 20 μ g protein) of each sample were Western blotted. The data shown is from one of two similar experiments.

Figure 2.

Effect of phospholipids on *rahU* gene expression in *P. aeruginosa*.

Effect of lysoPC on *rahU*⁺-*p-lacZ* promoter activity: Fig. 2A shows β -gal activity in *P. aeruginosa* cells untreated (□—□), or treated with 40 μ M lysoPC (▲—▲). Significant differences between control and lysoPC-treated *P. aeruginosa rahU*⁺-*p-lacZ*-b cells (* *p* 0.01, n=3) were observed in *rahU*⁺ promoter- β -gal activity at 8 and 11 h of growth.

Effect of OxPAPC and PAPC on *rahU*⁺-*p-lacZ* promoter activity: Fig. 2B shows β -gal activity in *P. aeruginosa* cells untreated (□—□), or treated with 50 μ g/ml of Ox-PAPC (△—△) or PAPC (◆—◆). Significant differences between control and Ox-PAPC or PAPC - treated *P. aeruginosa rahU*⁺-*p-lacZ*-b cells (* *p* 0.01, n=3) were observed in *rahU*⁺ promoter- β -gal activity at 8 and 11 h of growth.

Effect of AA on *rahU*⁺-*p-lacZ* promoter activity: Fig. 2C shows β -gal activity in *P. aeruginosa* cells untreated (□—□), or treated with 50 μ g/ml AA (○—○) or 50 μ g/ml AA + 50 μ g/ml lysoPC (■—■). Significant differences (* *p* 0.01) were observed in *rahU*⁺ promoter- β -gal. activity at 6, to 11 h of growth between control and AA -treated *P. aeruginosa rahU*⁺-*p-lacZ*-b cells. n=3. This inhibitory activity was restored by lysoPC.

Fragmentation of PAPC by treatment with PLA₂ to generate AA and/or lysoPC
Fig. 2D shows TLC analysis of various phospholipids. PAPC was treated with PLA₂ to release lysoPC and AA. PAPC was allowed to undergo auto-oxidation (mock PAPC) as described in Materials and Methods. TLC plates were loaded with Mock PAPC (lane 1), PLA₂ treated PAPC (lane 2), Standard lysoPC and AA (lane 3), standard PAPC (lane 4) and standard Ox-PAPC (lane 5).

Effect of free and complexed lysoPC on *rahU*⁺-*p-lacZ* promoter activity:

Fig. 2E shows β -gal activity in *rahU*⁺-*p-lacZ* *P. aeruginosa* cells that were exposed to 50 μ g/ml PAPC (△—△) or 50 μ g/ml AA (○—○), used to obtain independent base line values (control). The cells were also exposed to the mock PAPC (▲—▲) and PLA₂ treated PAPC (*—*) which was prepared as described for Fig. 4D. Significant differences between control (PAPC or AA), and PLA₂ or mock treated PAPC were seen at time periods of 6 and 10 h (**p* 0.01, n=3).

Figure 3A

Figure 3B

Figure 3.

Figure 3A. Biofilm formation is augmented in *rahU*⁻ cells. Biofilm formation was assessed in *P. aeruginosa* strains which were grown on Tryptic soya agar plate, (TSA experiments 1–4) or blood agar plates (BA, experiments 5–7). Both *P. aeruginosa* strains (*rahU*⁺ and *rahU*⁻) were grown separately in LB media for biofilm assay as described in Materials and Methods. Results are shown as mean ± SEM (n = 3). In all experiments, the biofilm formation in *rahU*⁺ and *rahU*⁻ cells were compared using a 2-sided *t*-test for independent samples with pooled variance method used for equal variances and the Satterthwaite approximation for unequal variances. To ensure that the overall type I error was preserved as 0.05, the Bonferroni correction was used, which can be declared statistically significant at

the 0.007 level for each test. Significance values obtained for each experiment were: Experiment #1, p 0.0043; #2, p 0.0001; #3, p 0.018; #4, 0.0001; #5, p 0.0137; #6, p 0.0434; #7, p 0.074. Additionally, the inverse chi-square method of Fisher was used to test for significance of the combined results. This method was applied to the four TSA experiments (p 0.0001) and the three blood agar primed experiments (p 0.0027). The seven combined experiments also showed statistically significant differences (p 0.0001) between $rahU^+$ and $rahU^-$ mutant cells.

Figure 3B. Biofilm formation is augmented by PAPC and AA in $rahU^+$ cells, but not in $rahU^-$ cells. Both *P. aeruginosa* strains ($rahU^+$ and $rahU^-$) were grown separately in LB in the absence or presence of 40 μ M lysoPC, 50 μ M PAPC or 50 μ M AA for 24 h and subjected to biofilm assays as described in Materials and Methods. Data is expressed as Mean $\pm$ SEM where $n=4$ to 6 for each experiment. Significance levels were calculated using students t test. The p values between untreated $rahU^+$ cells and those treated with lysoPC, PAPC and AA were p 0.2; p 0.001; p 0.001 respectfully. The p values were differentiated between untreated ($rahU^-$ cells) and those treated with lysoPC, PAPC and AA were p 0.03; p 0.3; p 0.2, respectively (compared with untreated controls).

Figure 4.

Schematic diagram showing pleotropic functional role of *rahU* in *P. aeruginosa* and its role in microbial sensing. *P. aeruginosa* produces a hemolysin like protein that is constitutively generated in the inner-membrane and extracellular medium during bacterial growth [25]. Host derived eukaryotic phospholipids such as PAPC, lysoPC, Ox-PAPC and AA moderate innate immunity and inflammation. LysoPC not only binds to the bacterial protein RahU, but can also augment *rahU* promoter activity and protein accumulation as indicated by arrows in PA0122 positive - *P. aeruginosa* 383 (*rahU*⁺) cells. There is no significant effect of lysoPC on biofilm formation in *rahU*⁺ cells as compared to their respective basal levels. Phospholipids (IPL) such as PAPC and AA that decrease *rahU* promoter activity augment biofilm accumulation in *rahU*⁺ cells. Abrogating the expression of *rahU* by insertion mutations to generate *rahU*⁻ cells spontaneously increases the basal levels of biofilm formation in *rahU*⁻ cells, when compared the basal levels seen in *rahU*⁺ cells. However, unlike the *rahU*⁺ cells, the *rahU* deficient *rahU*⁻ cells demonstrate no significant PL dependent augmentation of biofilm. Thus *rahU* gene was a prerequisite for modulation of biofilm formation induced by phospholipids. In summary, these experiments show that *rahU* gene and/or its protein not only specifically function as a ligand to host derived phospholipids such as Ox-LDL and lysoPC [25], but the experiments also show different functional attributes, which include: (a) *rahU* gene acts as a sensor and or receptor to host derived phospholipids and modulates *rahU*'s gene expression depending on the type of phospholipids; and (b) in turn *rahU* modulates biofilm formation.